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Differential Cytostatic and Apoptotic Effects of Ecteinascidin-743 in Cancer Cells

TRANSCRIPTION-DEPENDENT CELL CYCLE ARREST AND TRANSCRIPTION-INDEPENDENT JNK AND MITOCHONDRIAL MEDIATED APOPTOSIS*

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Consuelo Gajate, Feiyun An, and Faustino Mollinedo‡

From the Centro de Investigación del Cáncer, Instituto de Biología Molecular y Celular del Cáncer, Consejo Superior de Investigaciones Científicas-Universidad de Salamanca, Campus Miguel de Unamuno, E-37007 Salamanca, Spain

We have found that ecteinascidin-743 (ET-743) inhibited cell proliferation at 1–10 ng/ml, leading to S and G_2/M arrest and subsequent apoptosis, and induced early apoptosis without previous cell cycle arrest at 10–100 ng/ml in cancer cells. ET-743-mediated apoptosis, did not involve Fas/CD95. ET-743 induced c-Jun NH_2-terminal kinase (JNK) and caspase-3 activation, and JNK and caspase inhibition prevented ET-743-induced apoptosis. ET-743 failed to promote apoptosis in caspase-3-deficient MCF-7 cells, further implicating caspase-3 in its proapoptotic action. Overexpression of *bcl-2* by gene transfer abrogated ET-743-induced apoptosis, but cells underwent cell cycle arrest. ET-743 triggered cytochrome *c* release from mitochondria that was inhibited by Bcl-2 overexpression. Inhibition of transcription or protein synthesis did not prevent ET-743-induced apoptosis, but abrogated ET-743-induced cell cycle arrest. Microarray analyses revealed changes in the expression of a small number of cell cycle-related genes (*p21*, *GADD45A*, *cyclin G2*, *MCM5*, and histones) that suggested their putative involvement in ET-743-induced cell cycle arrest. These data indicate that ET-743 is a very potent anticancer drug showing dose-dependent cytostatic and proapoptotic effects through activation of two different signaling pathways, namely a transcription-dependent pathway leading to cell cycle arrest and a transcription-independent route leading to rapid apoptosis that involves mitochondria, JNK, and caspase-3.

Ecteinascidin-743 (ET-743)[1] is a marine-derived compound isolated from the marine tunicate *Ecteinascidia turbinata* (1, 2), with a potent cytotoxic activity against a variety of tumors *in vitro* and *in vivo* (3–5). The preclinical *in vivo* experiments with ET-743 showed cytotoxic activity of the drug when administered at $\mu g/m^2$ dosages, yielding nanomolar plasma concentrations (6, 7). Current phase II clinical trials in Europe and the United States indicate that ET-743 represents a highly promising antitumor agent. However, the mechanism by which ET-743 exerts its anticancer activity remains to be elucidated. ET-743 has been reported to bind to the minor groove of DNA (8, 9), bending DNA toward the major groove (10). DNA-bound ET-743 appeared to modify the interaction between DNA and several transcription factors (11, 12). Also, at high concentrations ET-743 and the related synthetic drug phthalascidin, have been reported to target topoisomerase I (13, 14). However, the relevance of these actions for the antitumor activity of ET-743 can be questioned as they are evidenced at drug concentrations much higher than those required for achieving its antitumor effect. On the other hand, ET-743-treated cells have been reported to accumulate in S and G_2/M phases (15–18).

To elucidate the mechanism underlying the anticancer effect of ET-743, we investigated the putative role of apoptosis in ET-743 action as an explanation for its cytotoxic effect. In this work we have found evidence for the induction of c-Jun NH_2-terminal kinase (JNK)-, mitochondria-, and caspase-3-mediated apoptosis in human cancer cells by ET-743 in a dose- and time-dependent way. Also, at very low concentrations, ET-743 is able to inhibit cell proliferation, accumulating cells in S or G_2/M, before promoting apoptosis after prolonged incubations. Our findings indicate that Bcl-2 overexpression is able to block the apoptotic effects of ET-743, preserving its actions on cell cycle. Cell cycle arrest induced by ET-743 was transcription-dependent, but ET-743-induced apoptosis was independent of transcription and of Fas/CD95. ET-743 had a rather weak effect on gene expression, but changes in the expression of a small number of genes seemed to account for the ET-743-promoted cell cycle arrest. Thus, ET-743 exerts two major concentration-dependent effects, namely cytostatic and proapoptotic, when used at concentrations (nM) required for the *in vivo* ET-743 anticancer action; therefore, these actions can account for the antitumor properties of the marine compound. Our data indicate that ET-743-induced cell cycle arrest and apoptosis result from the activation of two different signal transduction pathways.

EXPERIMENTAL PROCEDURES

Reagents—ET-743 was obtained from PharmaMar (Madrid, Spain) and prepared as a 1 mg/ml stock solution dissolved in ethanol and kept at −20 °C. Before use, the drug was diluted freshly in double-distilled sterile water to the desired concentrations. Dulbecco's modified Eagle's medium and RPMI 1640, fetal calf serum, antibiotics, and L-glutamine were purchased from Invitrogen. Rabbit polyclonal antiserum against human caspase-3 was from PharMingen (San Diego, CA). Mouse monoclonal antibody C2.10 against human poly(ADP-ribose) polymerase (PARP) was purchased from Enzyme Systems Products (Livermore, CA). Mouse monoclonal antibody Ab-1 against Bcl-2, and the JNK inhibitor SP600125 were from Calbiochem (Cambridge, MA). The

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‡ To whom correspondence should be addressed: Centro de Investigación del Cáncer, Instituto de Biología Molecular y Celular del Cáncer, Consejo Superior de Investigaciones Científicas-Universidad de Salamanca, Campus Miguel de Unamuno, E-37007 Salamanca, Spain. Tel.: 34-923-294806; Fax: 34-923-294795; E-mail: fmollin@usal.es.

[1] The abbreviations used are: ET-743, ecteinascidin-743; FasL, Fas ligand; GST, glutathione *S*-transferase; JNK, c-Jun NH_2-terminal kinase; PARP, poly(ADP-ribose) polymerase; z-D-dbmk, z-Asp-2,6-dichlorobenzoyloxymethylketone; HEL, human erythroleukemia.

caspase inhibitor z-Asp-2,6-dichlorobenzoyloxymethylketone (z-D-dbmk) and the recombinant human Fas ligand (FasL) were from Alexis (Läufelfingen, Switzerland). Acrylamide, bisacrylamide, ammonium persulfate, and *N*,*N*,*N*′,*N*′-tetramethylethylenediamine were from Bio-Rad (Richmond, CA). All other chemicals were from Merck (Darmstadt, Germany) or Sigma.

Cells and Culture Conditions—The following human leukemic cell lines were used: the acute myeloid leukemia HL-60 cell line; the T lymphoid Jurkat cell line, derived from an acute T-cell leukemia patient; the Bcr-Abl-positive K562 cell line, derived from a chronic myelogenous leukemia blast crisis patient; the Fas-resistant T-lymphoid RAPO leukemic cell line (a kind gift of Dr. P. H. Krammer, German Cancer Research Center, Heidelberg, Germany); and the erythroleukemia HEL cell line. These cells were grown in RPMI 1640 supplemented with 10% (v/v) heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 units/ml penicillin, and 24 μg/ml gentamicin. The human breast cancer cell line MCF-7 and the human epithelioid cervix adenocarcinoma HeLa cell line were grown in Dulbecco's modified Eagle's medium supplemented with fetal calf serum and antibiotics as above. Cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air.

HEL cells were transfected by electroporation with the SFFV-Neo expression vector containing the human *bcl-2* open reading frame driven by the long terminal repeat of the splenic focus-forming virus (pSFFV-*bcl-2*) as described previously (19) and selected by growth in the presence of 1 mg/ml G418. As a control, transfection was performed with empty SFFV-Neo plasmid.

ET-743 was added to the cell cultures at the concentrations and for the times indicated in the respective figures. Agents used to examine their action on ET-743-dependent apoptosis were added 20–30 min before ET-743 treatment. Cell growth was measured by cell counting and by [^{3}H]thymidine incorporation as described previously (20).

Apoptosis Assays—To assess apoptosis, fragmented DNA was isolated, analyzed by electrophoresis on 1% (w/v) agarose gels, and stained with ethidium bromide as described previously (20, 21). The induction of apoptosis was also monitored as the appearance of the sub-G_1 peak in cell cycle analysis (22) using a BD Biosciences FACSCalibur flow cytometer (San Jose, CA). Quantitation of apoptotic cells was calculated as the percentage of cells in the sub-G_1 region (hypodiploidy) in cell cycle analysis.

The possible implication of Fas/FasL interaction in ET-743-induced apoptosis was evaluated by using the blocking anti-Fas SM1/23 IgG_{2b} monoclonal antibody (Bender, Vienna, Austria) as described previously (23). Experiments performed with mock irrelevant isotype immunoglobulins had no effect.

Western Blot Analysis—About 10^7 cells were pelleted by centrifugation, washed with phosphate-buffered saline, lysed and subjected to Western blot analysis as described previously (24). Proteins (20 μg) were separated through sodium dodecyl sulfate (SDS)-polyacrylamide gels under reducing conditions, transferred to nitrocellulose filters, blocked with 5% powder defatted milk, and incubated overnight with the corresponding antibodies. Signals were developed using an enhanced chemiluminescence (ECL) detection kit (Amersham Biosciences, Aylesbury, UK).

Solid Phase JNK Assay—Protein kinase assays were carried out using a fusion protein between GST and c-Jun (amino acids 1–223) as a substrate for JNK, as described previously (22, 25). Cells (3–5 $\times$ 10^6) were resuspended in 200 μl of extract buffer (25 mM HEPES, pH 7.7, 0.3 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.1% Triton X-100, 20 mM β-glycerophosphate, 0.1 mM Na_3VO_4, 0.5 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml aprotinin). Cells were incubated for 30 min in continuous rotation at 4 °C and then microfuged at 12,000 rpm for 10 min. Pellets were discarded, and the supernatants, representing cell extracts, were diluted with 600 μl of dilution buffer (20 mM HEPES, pH 7.7, 0.1 mM EDTA, 2.5 mM $MgCl_2$, 0.05% Triton X-100, 20 mM β-glycerophosphate, 0.1 mM Na_3VO_4, 0.5 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml aprotinin). Mixtures were incubated for 10 min on ice and then microfuged at 12,000 rpm for 10 min. The cell extracts were mixed with 20 μl of a suspension in dilution buffer of glutathione-agarose beads to which GST-c-Jun was freshly bound. Mixtures were rotated overnight at 4 °C in an Eppendorf tube and pelleted by centrifugation at 12,000 rpm for 1 min. After four 1-ml washes in dilution buffer containing 50 mM NaCl to remove kinases that have a weaker affinity to bind c-Jun-(1–223) than JNK, the pelleted beads were resuspended in 30 μl of kinase buffer (20 mM HEPES, pH 7.7, 2 mM dithiothreitol, 20 mM β-glycerophosphate, 20 mM $MgCl_2$, 0.1 mM Na_3VO_4, 20 μM ATP) and incubated with 4 μCi of [γ-^{32}P]ATP. After 1 h at 30 °C, the reaction was terminated by washing with dilution buffer containing 50 mM NaCl and microfugation at 12,000 rpm for 1 min. Then the beads were boiled with 10 μl of 5 $\times$ SDS-polyacrylamide gel sample buffer to elute the phosphorylated proteins, which were subsequently resolved in a 10% SDS-polyacrylamide gel, followed by autoradiography. These conditions have been shown to enable specific binding of JNK to the c-Jun NH_2-terminal domain (25).

Cytochrome c Release Measurement—Release of cytochrome *c* from mitochondria to cytosol was analyzed by Western blot as previously described (26). Briefly, cells (4 $\times$ 10^6) were harvested by centrifugation, washed once with ice-cold phosphate-buffered saline, and gently lysed (by pipetting up and down 5 times) for 30 s in 50 μl of ice-cold buffer containing 250 mM sucrose, 1 mM EDTA, 0.05% digitonin, 25 mM Tris, pH 6.8, 1 mM dithiothreitol, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 μg/ml aprotinin, 1 mM benzimidine, and 0.1 mM phenylmethylsulfonyl fluoride. Lysates were centrifuged at 12,000 $\times$ *g* at 4 °C for 2 min to obtain the supernatants (cytosolic extracts free of mitochondria) and the pellets (fraction that contains mitochondria). Supernatants (40 μg of protein) and pellets (40 μg of protein) were electrophoresed on 15% SDS-polyacrylamide gels and analyzed by Western blot using anti-cytochrome *c* antibody (7H8.2C12, from PharMingen) and cytochrome *c* oxidase subunit II antibody (12C4-F12, from Molecular Probes, Eugene, OR) and ECL.

Affymetrix Microarray Analysis—Total RNA was isolated using a RNeasy kit (Qiagen, Valencia, CA). RNA integrity was assessed with an AGILENT 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany) using a RNA Nano LabChip (Agilent Technologies). Double-stranded cDNA was synthesized from 15 μg of total RNA by means of the SuperScript™ double-stranded cDNA synthesis kit (Invitrogen, San Diego, CA) with oligo$(dT)_{24}$ primer containing T7 RNA polymerase promoter. *In vitro* transcription was carried out by means of the Bioarray High Yield RNA Transcript Labeling kit (Enzo Diagnostics, Farmingdale, NY) with biotinylated cytidine triphosphate and uridine triphosphate. The biotin-labeled complementary RNA (cRNA) was purified with an RNeasy column and fragmented at 94 °C for 35 min in fragmentation buffer (40 mM Tris acetate, pH 8.1, 100 mM potassium acetate, 30 mM magnesium acetate). Integrity of cDNA, cRNA, and fragmented cRNA was assessed by electrophoresis of the samples on 1% agarose gels.

Microarray RNA analysis was performed according to the manufacturer's protocol using the Human Genome U95Av2 GeneChip (Affymetrix), which represents 12,625 human genes. Scanned output files were visually inspected for hybridization artifacts and then analyzed with the Affymetrix Microarray Suite 5.0 software. Arrays were scaled to an average intensity of 100 and analyzed independently. Genes were considered up-regulated or down-regulated if the expression was changed at least two-fold from the control. Only reliable and consistent mean values from four independent experiments with an appropriate standard deviation were considered. Data with low signal intensity, high background, and high variability among experiments were eliminated.

Statistical Analyses—Unless otherwise indicated, the results given are the mean (± S.D.) of the number of experiments indicated.

RESULTS

Dose-response and Time Course Effects of ET-743 on HL-60 and HeLa Cells—Human acute myeloid leukemia HL-60 cells were incubated with ET-743 for different periods of time at concentrations ranging from 0.1–100 ng/ml (0.131–131 nM, ET-743 mol. wt. is 761) and analyzed by flow cytometry (Figs. 1 and 2). No significant effect on cell cycle was observed at doses of 0.1 ng/ml ET-743. Incubation with 1 ng/ml ET-743 resulted in accumulation of cells in G_2/M (Figs. 1 and 2) and blockade of cell proliferation (>98% inhibition after 24 h treatment) (data not shown). About 50% of the cells were arrested at G_2/M with 4n content of DNA after 24-hour incubation (Figs. 1 and 2). This G_2/M arrest eventually led to cell death as protracted incubations with 1 ng/ml ET-743 induced a weak apoptotic response (about 11% apoptosis after >72 h of treatment). Treatment of HL-60 cells with 10 or 100 ng/ml ET-743 resulted in the rapid appearance of cells with a DNA content less than G_1, characteristic of apoptotic cells (Figs. 1 and 2), with no previous changes in cell cycle (Fig. 1, and data not shown).

Similar results were obtained when human cervix carcinoma HeLa cells were incubated with ET-743 (Fig. 3). Incubation with 1 ng/ml ET-743 resulted first in an increase of cells in S

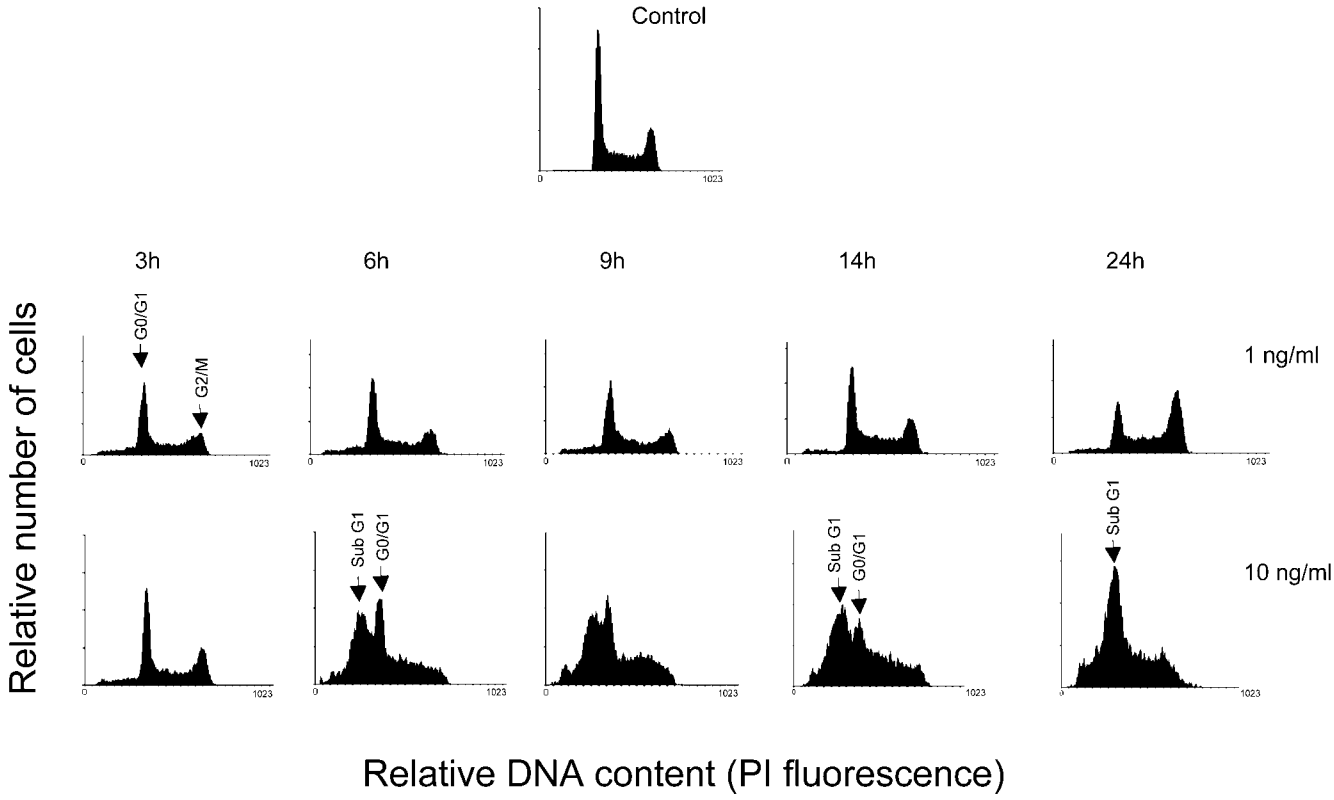

FIG. 1. **Time course of the effects of ET-743 on HL-60 cell cycle distribution.** HL-60 cells were untreated or treated with 1 or 10 ng/ml ET-743 for the times indicated, and their DNA content was analyzed by fluorescence flow cytometry. The position of the sub-G_1 peak (hypodiploidy), integrated by apoptotic cells, as well as the G_0/G_1 and G_2/M peaks are indicated by *arrows*. The experiment was repeated four times and representative histograms are shown.

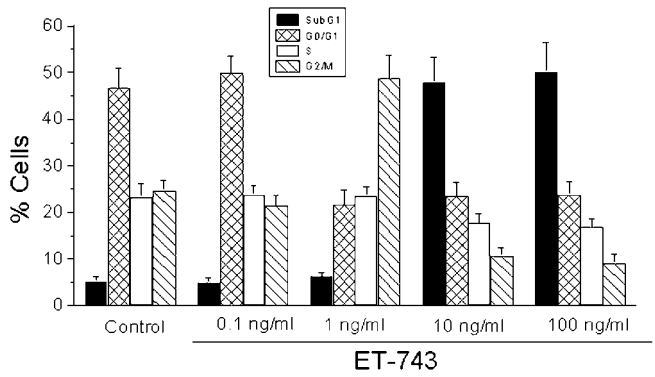

FIG. 2. **Dose response of the ET-743-induced effect on cell cycle in HL-60 cells.** Cells were incubated with increasing concentrations of ET-743 for 24 h, and the proportion of cells in each phase of the cell cycle was quantitated by flow cytometry. Cells in the sub-G_1 region represent apoptotic cells. Untreated control cells were run in parallel. Data are shown as means of four independent experiments ± S.D.

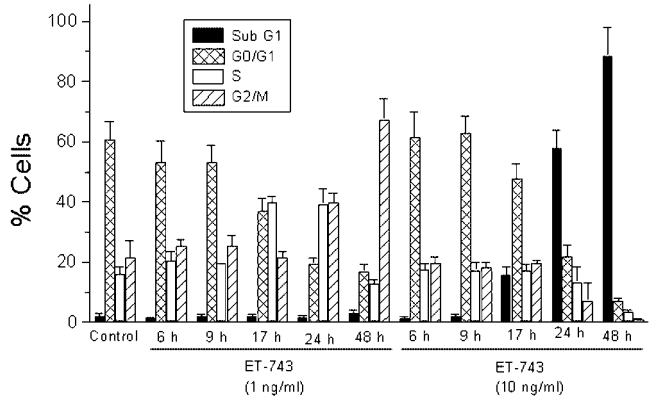

FIG. 3. **Time course of the effect of ET-743 on HeLa cell cycle distribution.** HeLa cells were incubated with 1 or 10 ng/ml ET-743 for the indicated times, and the proportion of cells in each phase of the cell cycle was quantitated by flow cytometry. Cells in the sub-G_1 region represent apoptotic cells. Untreated control cells were run in parallel. Data are shown as means of four independent experiments ± S.D.

phase and then in accumulation of cells in G_2/M (Fig. 3), with a blockade of cell proliferation (100% inhibition after 24 h treatment) (data not shown). No apoptotic response was elicited by 1 ng/ml ET-743 after 48 h treatment (Fig. 3). The effect on cell cycle was not reversed after drug washout, and cells eventually underwent apoptosis (about 12%, after >72 h of ET-743 washout). Incubation of HeLa cells with 10 (Fig. 3) or 100 (data not shown) ng/ml ET-743 resulted in a time-dependent accumulation of apoptotic cells with no previous changes in cell cycle.

Internucleosomal DNA Degradation Induced by ET-743—To demonstrate that ET-743 was able to induce an apoptotic response, we analyzed DNA degradation in ET-743-treated HL-60 and HeLa cells. Fig. 4 shows that ET-743, at concentrations of 10 or 100 ng/ml, induced the typical internucleosomal DNA fragmentation in multiples of 180–200 bp, a hallmark for apoptosis, in both tumor cell types.

Fas/CD95 Does Not Participate in ET-743-induced Apoptosis—Involvement of the Fas/CD95 receptor/ligand system has been proposed to mediate apoptosis induced by several anticancer drugs (27). The implication of Fas/FasL interaction in ET-743-induced apoptosis in the human acute T-cell leukemia Jurkat cells was evaluated by using the blocking anti-Fas SM1/23 monoclonal antibody, which abrogates Fas/FasL-mediated killing. Preincubation with SM1/23 anti-Fas antibody to-

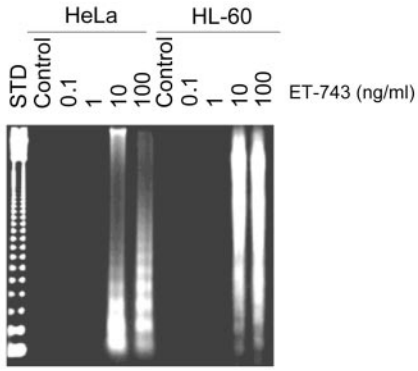

FIG. 4. **Internucleosomal DNA fragmentation induced by ET-743.** HeLa and HL-60 cells were incubated for 24 h with increasing concentrations of ET-743 and assayed for DNA fragmentation as described under "Experimental Procedures." Untreated control cells were run in parallel. Fragmented DNA from 6×10^5 cells were loaded in each lane of the agarose gel. A 123-bp DNA ladder was used as standard. Data are representative of three independent experiments performed.

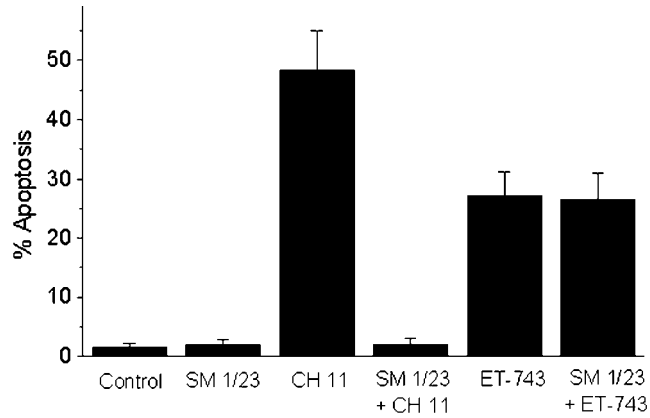

FIG. 5. **The Fas/FasL system is not involved in ET-743-induced apoptosis.** The effect of blocking anti-Fas monoclonal antibody on ET-743-induced apoptosis in T lymphoid leukemic Jurkat cells was analyzed. Cells were preincubated with blocking SM1/23 antibody for 30 min before addition of cytotoxic anti-Fas CH 11 antibody or 10 ng/ml ET-743 for 24 h. Untreated control cells and cells incubated only with SM1/23 antibody, CH 11 antibody, or ET-743 for 24 h were run in parallel. Apoptosis was then quantitated as a percentage of cells in the sub-G_1 region in cell cycle analysis. Data are shown as the means of three independent experiments ± S.D.

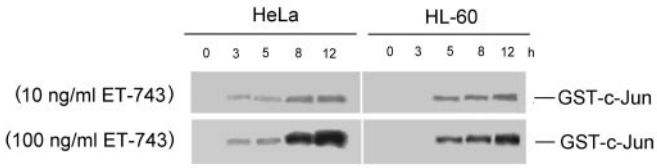

FIG. 6. **Time course of ET-743-induced JNK activation in HeLa and HL-60 cells.** Cells were treated with 10 or 100 ng/ml ET-743 for the times indicated and assayed for JNK activation. Untreated control cells were run in parallel in the same gel. The position of phosphorylated GST-c-Jun-1–223 (*GST-c-Jun*) is indicated. Experiments shown are representative of three performed.

tally prevented apoptosis induced by the agonistic cytotoxic CH 11 anti-Fas monoclonal antibody in Jurkat cells, but had no protective effect on ET-743-induced apoptosis (Fig. 5), suggesting that Fas/FasL interactions are not required for ET-743-induced apoptosis. We also found that ET-743 induced apoptosis in the Fas-resistant T-lymphoid RAPO leukemic cells (42.1 ± 4.3% and 76.8 ± 6.3% apoptosis after 24 h treatment with 10 and 100 ng/ml ET-743, respectively, $n = 3$). RAPO cells express Fas (about 60% cells positive for Fas), but incubation with 50 ng/ml CH 11 antibody or 50 ng/ml recombinant human FasL for 24 h did not induce apoptosis (less than 2.5% apoptosis, $n = 3$), indicating that Fas was non-functional in these cells. Furthermore, ET-743 induced apoptosis in the Fas-negative K562 leukemic cells (23), namely 19.2% and 25.3% apoptosis after 24 h treatment with 10 and 100 ng/ml ET-743, respectively. These results indicate that ET-743-induced apoptosis does not involve Fas/CD95.

ET-743-induced Apoptosis Is Mediated by Persistent JNK Activation—Because JNK activation has been suggested to be involved in the induction of apoptosis by different insults (28, 29), we examined whether ET-743 was able to activate JNK in HeLa and HL-60 cells through phosphorylation of its substrate c-Jun. To determine JNK activation, we used a GST fusion protein containing amino acids 1–223 of c-Jun (GST-c-Jun) as a substrate for JNK. ET-743 (10 and 100 ng/ml) induced a persistent JNK activation, measured as phosphorylation of GST-c-Jun, before the onset of apoptosis (Fig. 6). When ET-743 was used at 1 ng/ml, no JNK activation was detected before cell cycle arrest (data not shown). Curcumin (diferuloylmethane), an inhibitor of the JNK signaling pathway (30), inhibited both ET-743-induced JNK activation and apoptosis (Fig. 7, *A* and *B*). Furthermore, pretreatment of cells with the novel JNK inhibitor SP600125 (anthra[1,9-cd]pyrazol-6(2H)-one) (31) inhibited both ET-743-induced JNK activation and apoptosis (Fig. 7, *C* and *D*). These results suggest that the JNK signaling pathway is involved in ET-743-induced apoptosis.

Involvement of Caspase-3 Activation in ET-743-induced Apoptosis—We found that ET-743 induced caspase-3 activation in HL-60 and HeLa cells as assessed by cleavage of procaspase-3 into the p17 active form and cleavage of the typical caspase-3 substrate PARP, using a polyclonal anti-human caspase-3 antibody that recognized the 32-kDa proenzyme (pro-caspase-3) and the 17-kDa form of the active caspase-3, and the anti-PARP C2.10 monoclonal antibody that detected both the 116-kDa intact form and the 85-kDa cleaved form of PARP (Fig. 8*A*). The caspase-3 cleavage product p19 observed in Fig. 8*A* represents the p17 subunit plus the short caspase-3 prodomain, which is then slowly converted into the active p17 subunit (32). Incubation with the aspartate-based caspase inhibitor z-Asp-2,6-dichlorobenzoyloxymethylketone (z-D-dbmk) completely blocked PARP degradation (data not shown) and apoptosis in ET-743-treated HL-60 cells (Fig. 8*B*) and HeLa cells (data not shown). We also found that MCF-7 cells, which are deficient in caspase-3 because of a 47-bp deletion within exon 3 of the *caspase-3* gene (33), did not undergo apoptosis upon ET-743 treatment (4.1 ± 1.4% apoptosis in untreated MCF-7 cells *versus* 4.2 ± 1.7% apoptosis in MCF-7 cells treated with either 10 or 100 ng/ml ET-743 for 24 h, $n = 3$). These data support the involvement of caspase-3 in ET-743-induced apoptosis.

Inhibition of ET-743-induced Apoptosis by Overexpression of bcl-2—Bcl-2 regulates apoptosis, acting as suppressor (34). Thus, we analyzed whether ET-743-induced apoptosis was under Bcl-2 control in human erythroleukemia HEL cells. These cells were stably transfected with pSFFV-*bcl-2* (HEL-Bcl-2), containing the human *bcl-2* open reading frame, or with control pSFFV-Neo plasmid (HEL-Neo). Western blot analysis indicated that HEL-Neo cells expressed no Bcl-2, whereas a high expression of this protein was observed in HEL-Bcl-2 cells, as previously reported (19, 22). HEL-Neo cells were accumulated in S phase before undergoing apoptosis when treated with 10 ng/ml ET-743 and underwent apoptosis without any previous effect on cell cycle at 100 ng/ml ET-743 (Fig. 9*A*). Overexpression of Bcl-2 by gene transfer prevented ET-743-induced apoptosis, even after 72 h of treatment. But cells did not proliferate (100% inhibition in cell growth) and accumulated in S or G_0/G_1 when treated with 10 or 100 ng/ml ET-743, respectively (Fig.

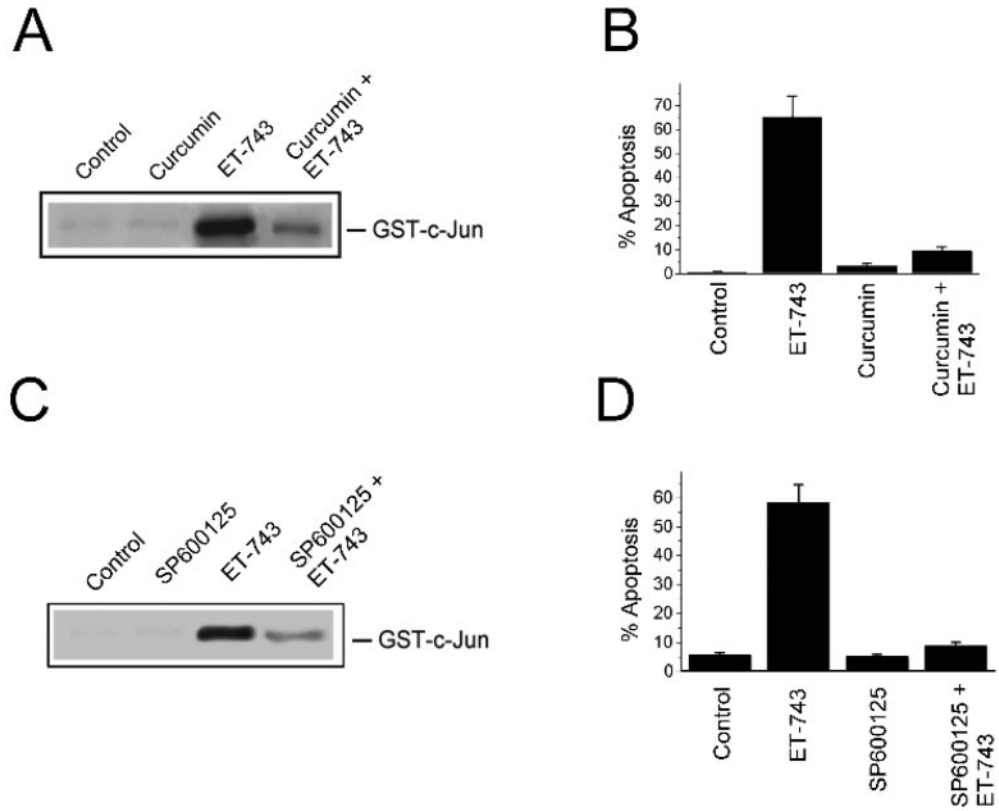

FIG. 7. **Effect of curcumin and SP600125 on ET-743-induced JNK activation and apoptosis.** HeLa cells were incubated in the absence or in the presence of curcumin (20 μM), ET-743 (100 ng/ml) or curcumin + ET-743 for 8 h and analyzed for JNK activation (*A*) or for 24 h and analyzed for apoptosis through fluorescence flow cytometry (*B*). HL-60 cells were incubated in the absence or in the presence of SP600125 (20 μM), ET-743 (10 ng/ml), or SP600125 + ET-743 for 8 h and analyzed for JNK activation (*C*), or for 24 h and analyzed for apoptosis through fluorescence flow cytometry (*D*). The position of phosphorylated GST-c-Jun-1–223 (*GST-c-Jun*) is indicated. Experiments shown are representative of three performed.

9*B*). Quantitative data showed that 65.0 ± 5.6% and 66.2 ± 5.0% (n = 3) of HEL-Bcl-2 cells treated for 72 h with 10 and 100 ng/ml ET-743 accumulated at S and G_0/G_1 phase, respectively, whereas 43.1 ± 3.7% and 38.1 ± 3.1% (n = 3) of untreated HEL-Bcl-2 cells were in G_0/G_1 and S phase, respectively.

ET-743 Induces Translocation of Mitochondrial Cytochrome c into the Cytosol—Because ET-743-triggered apoptosis was tightly controlled by Bcl-2, we asked whether ET-743 could trigger mitochondrial cytochrome *c* release, as Bcl-2 has been reported to interfere with cytochrome *c* release from mitochondria (34). As shown in Fig. 10, cytosolic cytochrome *c* levels markedly and rapidly increased after treatment of HL-60 cells with proapoptotic concentrations of ET-743 (100 ng/ml). Similar results were obtained also when cells were treated with 10 ng/ml ET-743 (data not shown). However, no cytochrome *c* release was detected when HL-60 cells were incubated with 1 ng/ml ET-743 for up to 12 h of treatment (data not shown), conditions that did not promote apoptosis. Overexpression of Bcl-2 in HEL cells prevented both apoptosis (Fig. 9) and mitochondrial cytochrome *c* release (data not shown). These results suggest that cytochrome *c* release from mitochondria is an early event in ET-743-induced apoptosis.

Inhibition of Protein Synthesis Prevents ET-743-induced Cell Cycle Arrest, but Not ET-743-induced Apoptosis—Because the cytotoxic activity of ET-743 has been previously related to its capacity to affect transcription (11, 12), we examined whether inhibition of macromolecule synthesis could affect the apoptotic action of ET-743. Pretreatment of HeLa cells with cycloheximide or actinomycin D, used at concentrations that inhibited protein and mRNA synthesis (Ref. 35 and data not shown), did not affect the apoptotic response induced by ET-743, but the cell cycle arrest following incubation with 1 ng/ml ET-743 was completely prevented (Fig. 11).

Changes in Gene Expression Induced by ET-743 During Cell Cycle Arrest—Because the effects of ET-743 on cell cycle arrest were caused by a transcription-mediated process (Fig. 11), we investigated the effects of ET-743 on gene expression by using Affymetrix Human Genome U95Av2 GeneChip oligonucleotide arrays. HeLa cells were treated with 1 ng/ml ET-743 for 48 h, under conditions that induced G_2/M arrest (69.2 ± 8.6% cells were arrested in G_2/M, n = 4) but no apoptosis, and their gene expression pattern was compared with untreated control HeLa cells. Table I lists 89 genes that were down- and up-regulated at least two-fold by drug treatment; 20 transcripts were down-regulated and 69 transcripts were up-regulated. Although the relevance of some of the mRNAs that were regulated by ET-743 treatment is unclear, we found significant changes in the expression of a number of cell cycle-related genes (histones, *MCM5*, *cyclin G2*, *GADD45A* and *p21*) that could explain the transcription-mediated effect of ET-743 on cell cycle arrest (Table I). Histones and *MCM5* were down-regulated in ET-743-treated cells, and histones have been found to play a role during the G_2/M transition (36). Reduced minichromosome maintenance (MCM) protein levels induce a delay in S phase and hamper cell cycle progression (37). ET-743 treatment up-regulated *cyclin G2*, *GADD45A* and *p21* transcripts, and *cyclin G2* has been reported to be up-regulated during late S phase (38) and during growth inhibition (39). *GADD45A* (40) and *p21* (41) induce G_2 cell cycle arrest. Thus, the changes in the expression of these five cell cycle-related genes could account for the observed effects of ET-743 on cell cycle, namely a delay in S phase and an arrest in G_2/M phase.

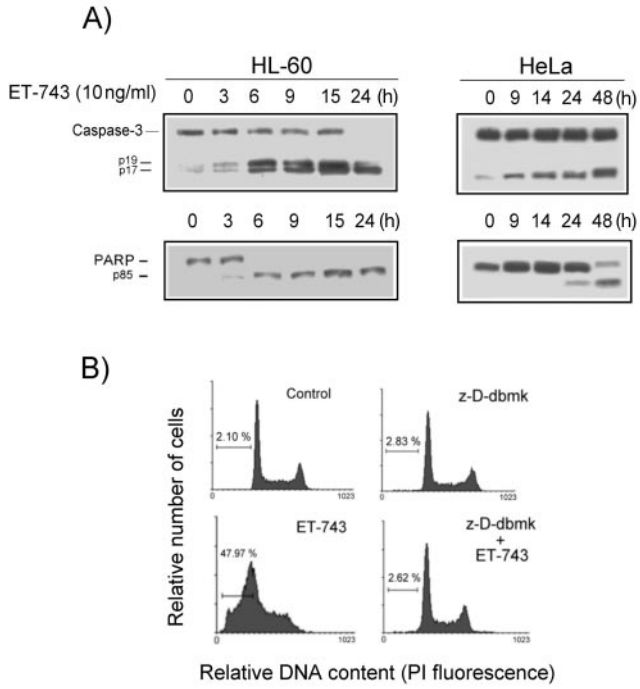

FIG. 8. **Involvement of caspase-3 in ET-743-induced apoptosis.** *A*, time course of caspase-3 and PARP cleavage in ET-743-treated HL-60 and HeLa cells. Cells were treated with ET-743 for the indicated times and analyzed by immunoblotting with anti-caspase-3 and anti-PARP antibodies. The migration positions of full-length caspase-3 and the p19 and p17 cleavage products, as well as of full-length PARP and its cleavage product p85 are indicated. *B*, caspase inhibition prevents apoptosis in ET-743-treated cells. HL-60 cells were incubated with 10 ng/ml ET-743 in the absence or in the presence of 50 μM z-Asp-2,6-dichlorobenzoyloxymethylketone (z-D-dbmk) for 24 h, and then analyzed for DNA degradation by flow cytometry. Untreated control cells and cells treated only with the caspase inhibitor were also run in parallel. The percentage of cells with a DNA content less than G_1, representing apoptotic cells, is indicated in each histogram. Data shown are representative of three experiments performed.

DISCUSSION

The data reported here demonstrate that ET-743 is able to induce an apoptotic response leading to the internucleosomal DNA degradation in human cancer cells. This apoptotic activity of ET-743 can account for the previously reported cytotoxic effects exerted by this antitumor agent (1–5). Takahashi *et al.* (42) has recently reported that a combination of doxorubicin and ET-743 induced a weak apoptotic response, based on nuclei morphology, in fibrosarcoma cell lines, but no apoptosis was detected following treatment with ET-743 alone. Here, we show compelling evidence for the proapoptotic activity of ET-743 itself in different cell lines by using a combination of different techniques. Our data also demonstrate that the apoptotic effect of ET-743, leading to internucleosomal DNA degradation, is independent of transcription and of Fas/CD95 but involves JNK, mitochondria, and caspase-3. The ability of ET-743 to rapidly induce cytochrome *c* release from mitochondria as well as the abrogation of ET-743-induced apoptosis and mitochondrial cytochrome *c* release by Bcl-2 overexpression indicates a major role for mitochondria in the induction of apoptosis by this marine antitumor drug. Both JNK activation and mitochondrial cytochrome *c* release constitute early events in ET-743-induced apoptosis as they are detected in the first 6 h of treatment. Furthermore, we found that ET-743 exerts two major dose-dependent actions on cancer cells: (*a*) transcription-dependent growth arrest with an accumulation of cells in S and G_2/M when used at 1–10 ng/ml, and (*b*) transcription-independent apoptosis when used at 10–100 ng/ml. These data explain the previous *in vitro* antiproliferative activity of the compound assessed by using metabolic assays, leading to the notion that ET-743 is a potent and effective antiproliferative drug at the ng/ml (nM, ET-743 mol. wt. is 761) range.

ET-743 has been shown to alkylate DNA, preferentially at GC-rich sequences (8, 9). Previous reports have shown that ET-743 inhibits at the μM range (>10 μM) the activity of various DNA-binding factors, including TBP, E2F, SRF, and NF-Y (11). High concentrations (μM range) of ET-743 have also been reported to inhibit topoisomerase I and to induce protein-linked DNA single-strand breaks (11, 13–15). However, the high concentrations required *in vitro* to detect these effects raise the question of the pharmacological relevance of such observations, as ET-743 is cytotoxic and pharmacologically active at much lower concentrations (nM range). In addition, the relevance of the action of ET-743 on topoisomerase I is questionable because this drug is equally active in cells lacking detectable topoisomerase I (15). Two recent studies reported that relatively low concentrations (10–50 nM) inhibited the transcriptional activation of cellular genes regulated by the NF-Y transcription factor, including MDR1, and possibly by the Sp1 transcription factor (12, 43). However, although ET-743 interaction with the DNA seems to be well characterized (8, 9), the molecular mechanisms by which such an interaction leads to growth arrest and cytotoxicity are not well understood. The data reported here, showing that pharmacologically relevant concentrations (nM) of ET-743 induce a potent apoptotic response, indicate that triggering of apoptosis can be the major action in ET-743 cytotoxicity.

We have also found that ET-743 induced an accumulation of cells in S phase and a G_2/M arrest when used at very low doses, in agreement with previous observations (15–18). It has been reported that ET-743 disorganizes the microtubule network (44), but this action was evidenced at very high drug concentrations (μM). We have not detected any effect of ET-743 on the microtubule network when pharmacologically active concentrations (1–10 ng/ml) of ET-743 were used.[2] Interestingly, we have found here that the action of ET-743 on cell cycle was dependent on transcription. To investigate the genes responsible for this transcription-dependent cell cycle inhibition, we carried out Affymetrix Microarray analyses of the RNA expression profiles in HeLa cells treated with doses of ET-743 that induced exclusively cell cycle arrest and no apoptosis, and compared these array data with those from untreated control HeLa cells. We calculated mean values from four different experiments, and only those with an acceptable standard deviation, after stringent analysis, were considered to be consistent and reliable figures. Although ET-743 has been reported to influence transcription (11, 12, 43), the number and extension of changes in gene expression induced by the drug was rather low. Carrying out parallel experiments of cell cycle and microarray analyses, we found that only 89 genes of the 12,625 genes analyzed were regulated in their expression (20 down-regulated genes and 69 up-regulated genes) when cells were treated under conditions that induced cell cycle arrest (1 ng/ml ET-743, 48 h). Interestingly, we found that ET-743 regulated the expression of five cell cycle-related genes that could explain the transcription-dependent effect of ET-743 on cell cycle, namely up-regulation of *cyclin G2*, *GADD45A* and *p21* transcripts, and down-regulation of histones and *MCM5*. Up- and down-regulation of the above genes have been reported to induce an increase in S phase and a G_2/M arrest in different systems (36–41), the same effects observed in ET-743-treated cells. Thus, these data prompt us to envisage a hypothesis

[2] F. An, C. Gajate, and F. Mollinedo, unpublished observations.

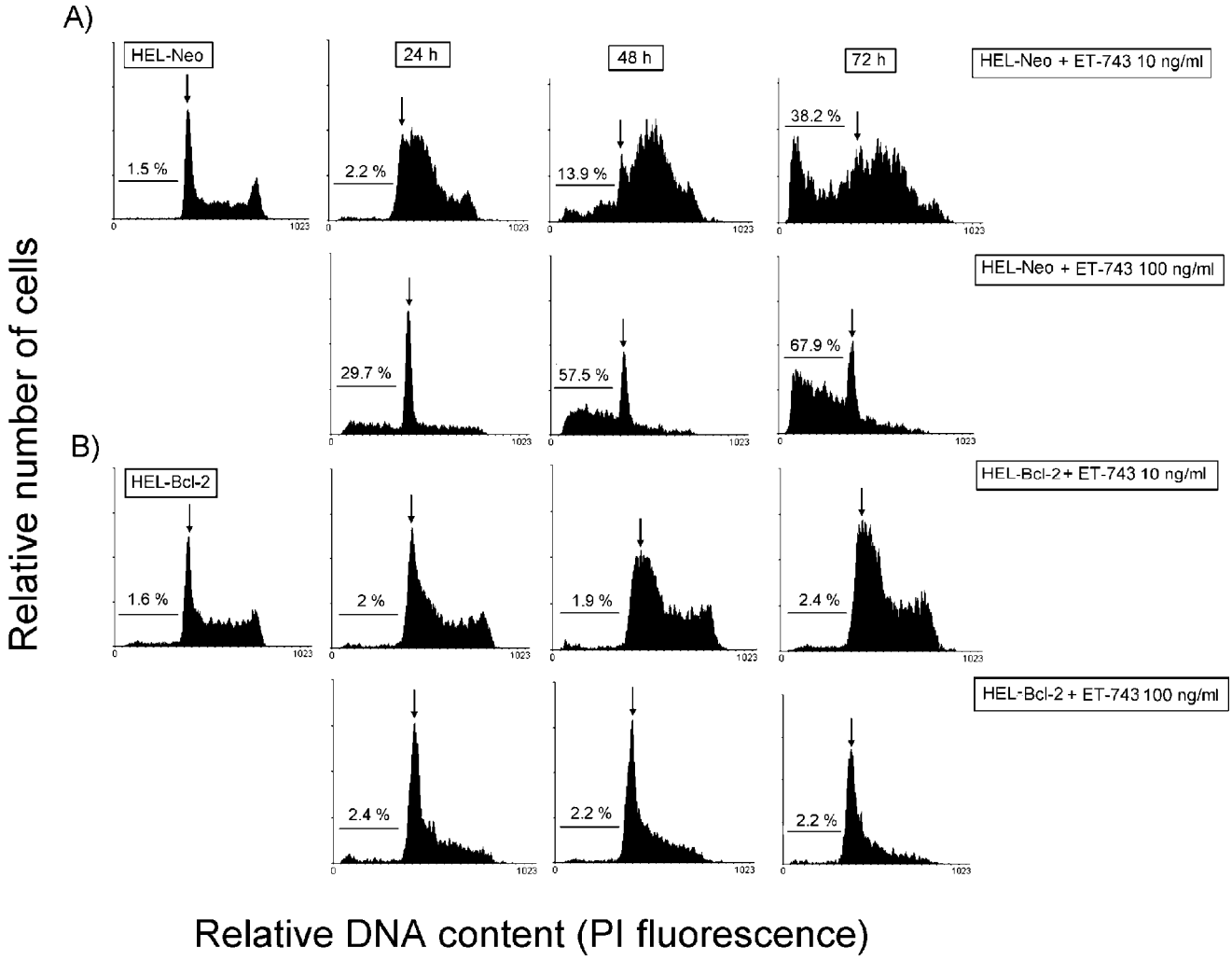

FIG. 9. **Prevention of ET-743-induced apoptosis by Bcl-2 overexpression.** The cell cycle and apoptotic effects of ET-743 on HEL-Neo (*A*) and HEL-Bcl-2 (*B*) cells are shown. The drug concentrations used and the incubation times are indicated. The DNA content of untreated control cells (*upper left* histograms in *A* and *B*) and cells treated with ET-743 was analyzed by fluorescence flow cytometry. The position of the G_0/G_1 peak is indicated by *arrows*. The percentage of cells with a DNA content less than G_1 (sub-G_1), representing apoptotic cells, is indicated in each histogram. Data shown are representative of three experiments performed.

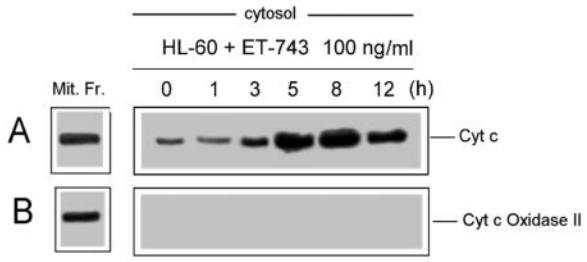

FIG. 10. **ET-743 triggers cytosolic accumulation of cytochrome *c*.** HL-60 cells treated with 100 ng/ml ET-743 were harvested at the indicated times, and cytosolic and mitochondrial proteins were analyzed by immunoblotting with anti-cytochrome *c* (*A*) or anti-cytochrome *c* oxidase II (*B*). Cytochrome oxidase serves as a marker for mitochondrial contamination of cytosolic extracts. A mitochondrial extract from untreated control cells (*Mit. Fr.*) was used as a positive control for cytochrome *c* and cytochrome oxidase. Data shown are representative of three experiments performed.

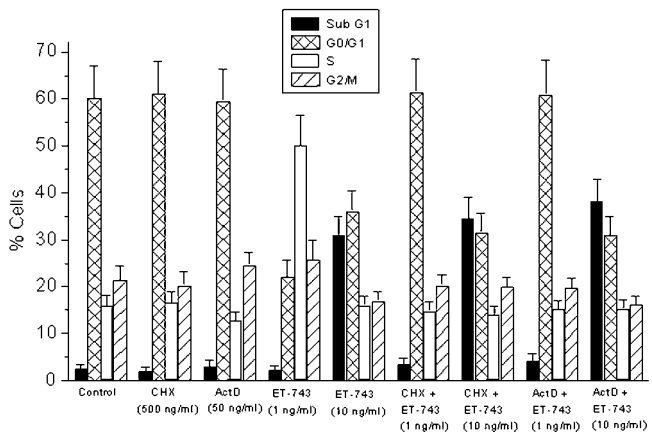

FIG. 11. **Effect of protein synthesis inhibition on the actions of ET-743 in cell cycle and apoptosis.** HeLa cells were preincubated for 20 min in the absence and in the presence of 500 ng/ml cycloheximide (*CHX*) or 50 ng/ml actinomycin D (*ActD*) and then treated with different concentrations of ET-743 for 20 h. The proportion of cells in each phase of the cell cycle was quantitated by flow cytometry. Cells in the sub-G_1 region represent apoptotic cells. Untreated control cells were run in parallel. Data are shown as the means of three independent experiments ± S.D.

where changes in the expression of the above genes may lead to the transcription-dependent cytostatic effects induced by ET-743. This represents a working hypothesis that must be tested in future experiments. In addition to the above genes, we also found that ET-743 regulated the expression of additional genes

TABLE I

Gene expression changes induced by ET-743 in HeLa cells

Genes related to cell cycle are labeled in **bold**. Data show the fold change values of genes in ET-743-treated HeLa cells compared to untreated control HeLa cells. Data are shown as mean values ± S.D. of four independent experiments. Listed here are genes that showed at least a 2-fold change in expression.

Accession no.	Fold change		Gene definition	Putative function
	Mean	S.D.		
AF042377	−6.0	2.9	GDP-mannose 4,6-dehydratase	metabolism
AF036715	−3.4	0.9	syntaxin 8	membrane traffic
AA255502	**−3.0**	**1.3**	**H4 histone family, member G**	**cell cycle; transcription**
AF097441	−2.9	0.4	phenylalanine-tRNA synthetase	protein biosynthesis
D64142	**−2.6**	**0.7**	**H1 histone family, member X**	**cell cycle; transcription**
X81889	−2.6	0.8	plakophilin 4	cell adhesion
X60484	**−2.6**	**0.8**	**H4 histone family, member E**	**cell cycle; transcription**
X69550	−2.5	0.7	Rho GDP dissociation inhibitor (GDI) alpha	signal transduction; cell adhesion
AF070598	−2.4	0.2	ATP-binding cassette, sub-family B (MDR/TAP), member (ABCB6)	drug resistance
Z75330	−2.2	0.9	stromal antigen 1 (SA-1)	unknown
X74795	**−2.1**	**0.3**	**minichromosome maintenance protein 5 (MCM5)**	**cell cycle; DNA replication**
U95006	−2.1	0.3	D9 splice variant A	unknown
AB020641	−2.1	0.6	PFTAIRE protein kinase 1 (PFTK1)	signal transduction
U28042	−2.0	0.2	DEAD/H box polypeptide 10 (RNA helicase) (DDX10)	transcription
AL021154	−2.0	0.2	EST	unknown
AF096870	−2.0	0.2	tripartite motif-containing 16 (TRIM16)	transcription
M83822	−2.0	0.2	beige-like protein (BGL)	membrane traffic
AJ006778	−2.0	0.2	down-regulated in metastasis (DRIM)	negative control of cell proliferation
AA195301	−2.0	0.2	hypothetical protein MGC2574	unknown
U63824	−2.0	0.2	TEA domain family member 4	transcription
M82809	2.0	0.2	annexin A4 (ANXA4)	membrane traffic
AI951946	2.0	0.2	EST	unknown
X74764	2.0	0.2	discoidin domain receptor family, member 2	cell adhesion; signal transduction
M15036	2.0	0.2	protein S (alpha)	blood coagulation
L06797	2.0	0.2	chemokine (C-X-C motif), receptor 4 (fusin)	cell-cell signaling
AF025887	2.0	0.2	glutathione S-transferase A4	stress response
AI017574	2.0	0.2	cysteine-rich protein 1	cell proliferation
L06845	2.0	0.2	cysteinyl-tRNA synthetase	protein biosynthesis
U41843	2.0	0.3	DR1-associated protein 1 (negative cofactor 2 alpha)	repression of transcription
U47414	**2.0**	**0.3**	**cyclin G2**	**cell cycle checkpoint**
V01512	2.0	0.3	c-fos	transcription
M77349	2.0	0.3	transforming growth factor, beta-induced, 68 kD	cell proliferation
L19182	2.0	0.3	insulin-like growth factor binding protein 7 (IGFBP7)	negative control of cell proliferation
M60974	**2.0**	**0.3**	**growth arrest and DNA-damage-inducible, alpha (GADD45A)**	**cell cycle arrest; DNA repair; apoptosis**
U28014	2.0	0.3	caspase-4	apoptosis
D16532	2.0	0.3	very low density lipoprotein receptor	metabolism
AL050002	2.0	0.3	DKFZp5640222	unknown
AJ001685	2.0	0.3	NKG2E	NK cell regulation
M14058	2.0	0.3	complement component C1r	complement; proteolysis
W72186	2.0	0.3	S100 calcium-binding protein A4	membrane traffic
X51405	2.0	0.3	carboxypeptidase E (CPE)	signal transduction; metabolism
AA131149	2.0	0.3	S100 calcium-binding protein P	membrane traffic
AI382123	2.0	0.5	EST	unknown
U15932	2.0	0.5	dual specificity phosphatase 5	heat shock response; signal transduction
X58536	2.0	0.5	major histocompatibility complex, class I, C	immune response regulation
M84526	2.0	0.5	D component of complement (adipsin)	complement; proteolysis
L00972	2.0	0.5	Human cystathionine-beta-synthase	metabolism
U51334	2.0	0.5	TAF15 RNA polymerase II	transcription
AI535653	2.0	0.5	sterol-C4-methyl oxidase-like (SC4MOL)	metabolism
M25915	2.0	0.5	clusterin	metabolism
U09510	2.0	0.5	glycyl-tRNA synthetase	protein biosynthesis
AL096717	2.0	0.5	DKFZp564P0662	unknown
AF023462	2.1	0.2	phytanoyl-CoA hydroxylase	metabolism
M59040	2.1	0.3	CD44 antigen	metastasis; homing
D50918	2.1	0.3	septin 6	signal transduction
L13463	2.1	0.3	regulator of G-protein signaling 2, 24kD	signal transduction
U60521	2.1	0.3	caspase-9	apoptosis
U57646	2.1	0.3	cysteine and glycine-rich protein 2	development; differentiation; cytoskeleton
X17042	2.1	0.3	proteoglycan 1, secretory granule	extracellular matrix
AB020662	2.1	0.3	golgin-67 (KIAA0855)	Golgi-targeted protein
D49950	2.1	0.3	interleukin 18 (IL18)	cell-cell signaling
D31885	2.1	0.4	ADP-ribosylation factor-like 6 interacting protein (KIAA0069)	unknown
AL080061	2.1	0.5	DKFZp56H182	unknown
AF000381	2.1	0.5	folate binding protein	metabolism
X76732	2.1	0.5	nucleobindin 2	DNA binding
D86425	2.1	0.7	nidogen 2 (NID2)	cell adhesion
U03106	**2.2**	**0.2**	**cyclin-dependent kinase inhibitor 1A (p21, Cip1)**	**cell cycle arrest**
AI445461	2.2	0.3	transmembrane 4 superfamily member 1 (TM4SF1)	cell proliferation
X13589	2.2	0.3	cytochrome P450	drug metabolism
AI885381	2.2	0.5	hypothetical protein MGC2650	unknown
D50840	2.2	0.5	UDP-glucose ceramide glucosyltransferase	metabolism
U40490	2.3	0.3	nicotinamide nucleotide transhydrogenase	energy pathways
X76534	2.3	0.3	glycoprotein (transmembrane) nmb	negative control of cell proliferation

TABLE I—*continued*

Accession no.	Fold change		Gene definition	Putative function
	Mean	S.D.		
X04430	2.3	0.3	interleukin 6 (IL6)	signal transduction
X05908	2.3	0.3	annexin A1 (ANXA1)	membrane traffic
M59465	2.3	0.3	tumor necrosis factor, alpha-induced protein 3 (TNFAIP3)	unknown
X04412	2.4	0.2	gelsolin	actin modification
L19871	2.4	0.2	activating transcription factor 3 (ATF3)	transcription
W47047	2.4	0.3	p8 protein (candidate of metastasis 1)	metastasis
AJ225089	2.4	0.3	2′-5′-oligoadenylate synthetase-like	metabolism
U78793	2.5	0.3	folate receptor alpha	metabolism
X92720	2.5	0.8	phosphoenolpyruvate carboxykinase 2	metabolism
S73591	2.7	0.8	thioredoxin interacting protein	unknown
M31452	2.9	0.4	complement component 4-binding protein, alpha (C4BPA)	complement; cell recognition
M13755	3.2	0.8	interferon-stimulated protein, 15 kDa	unknown
AJ011497	3.4	0.4	claudin 7	cell-cell communication
M27396	3.5	0.5	asparagine synthetase	metabolism
AI989422	4.4	0.7	fibrinogen, gamma polypeptide	metastasis; cell adhesion
X01703	5.0	0.9	tubulin, alpha 3	cytoskeleton

involved in cell proliferation and induced expression of proliferation repressors (Table I) that could also be involved in the transcription-dependent inhibition of cell cycle by ET-743. Martinez *et al*. (17) have recently reported an array-based gene expression study analyzing about 5,000 genes in breast carcinoma MDA-MB-435 cells and colon carcinoma HCT116 cells treated with 2 nM ET-743 for 12 h, showing that down-regulation of transcripts was predominant over the up-regulation of transcripts, with 54–61 down-regulated genes and 1–8 up-regulated genes. In agreement with our data, up-regulation of *p21* by ET-743 was consistently found in that study in both cell types. In addition we have found that at shorter drug incubation times (1 ng/ml ET-743, 24 h) ET-743 had a very weak effect on gene expression, considering mRNA levels modified in at least two-fold, and the predominant effect was down-regulation (25 down-regulated genes *versus* 3 up-regulated genes).[3] Although some similarities can be found between the array data reported by Martinez *et al.* (17) and our present microarray profiles, including the relatively low number of genes regulated by the drug, differences are also evident. These differences can be caused by the use of distinct cell lines (HeLa *versus* HCT116 and MDA-MB-435 cells), different experimental conditions (1 ng/ml–1.3 nM ET-743, 48 h *versus* 2 nM ET-743, 12 h), different arrays (Affymetrix HGU95Av2 *versus* HuGeneFL arrays), and different software to analyze the data. Concerning this last aspect, we have used the novel Affymetrix Microarray Suite 5.0 software that largely improves the reliability and consistency of the figures at the expense of a significant decrease in the fold change values obtained with previous software versions.

Fig. 12 depicts a scheme of the different effects exerted by ET-743 on cell cycle and apoptosis based on the present results. ET-743 inhibits cell proliferation at 1–10 ng/ml, leading to an accumulation of cells in the S or G_2/M phases after 24–48 h of treatment. ET-743 promotes apoptosis after 6–17 h of treatment at 10–100 ng/ml without any previous effect on cell cycle. Overexpression of Bcl-2 inhibited apoptosis induced by ET-743, but cells underwent cell cycle arrest and were unable to proliferate, suggesting that Bcl-2 blocks ET-743-induced apoptotic signaling but preserves the drug effects on cell cycle. Depending on the ET-743 dose used, overexpression of Bcl-2 in HEL cells arrested cells in S or G_0/G_1. Thus, ET-743 seems to be able to affect different cell cycle checkpoints in a dose-dependent way. Although the ET-743 antitumor action has previously been related to its effects on transcription (11, 12, 43), the lack of effect of actinomycin D and cycloheximide on ET-743-induced apoptosis indicates that ET-743 can induce cell death through a transcription-independent route.

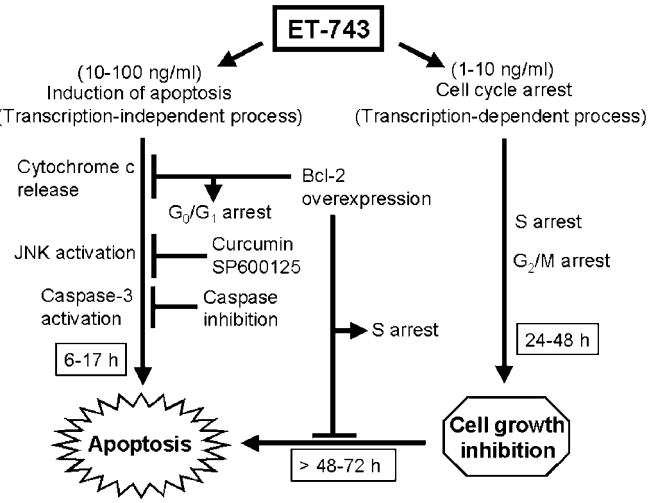

FIG. 12. **Anti-proliferative and proapoptotic actions of ET-743 on tumor cells.** This is a schematic diagram designed to portray the biological processes and biochemical events that we have detected in human cancer cells treated with different concentrations of ET-743. At low concentrations, ET-743 affects mainly cell cycle, leading to cell growth inhibition and eventually to late apoptosis after very prolonged incubation times. At higher concentrations ET-743 induces an early apoptotic response without any previous effect on cell cycle. Timings for the different processes elicited by ET-743 are shown in *boxes*. Overexpression of Bcl-2 inhibits mitochondrial cytochrome *c* release and prevents cells from entering into apoptosis, but cells undergo cell cycle arrest. Prevention of JNK or caspase activation also inhibits apoptosis. The hierarchical order of mitochondrial cytochrome *c* release, JNK activation, and caspase-3 activation remains to be established. Therefore, the order of appearance of these signaling events in the figure does not necessarily indicate the actual sequence of cause-effect events in the apoptotic signaling induced by ET-743.

The data reported here indicate that the apoptotic pathway triggered by 10–100 ng/ml ET-743 involves mitochondrial cytochrome *c* release, JNK activation, and caspase-3 activation. However, further studies must be completed to establish a hierarchy in their actions; therefore, the order of appearance of these three signaling routes in the model outlined in Fig. 12 does not necessarily indicate the actual sequence of cause-effect events or hierarchical order in the apoptotic signaling induced by ET-743. Recent reports suggest that JNK activation precedes mitochondrial cytochrome *c* release (45, 46). However, we have found that inhibition of JNK activation by either curcumin (30) or by the novel JNK inhibitor SP600125 (31) inhibited apoptosis, but potentiated cytochrome *c* release from mitochon-

[3] F. Mollinedo, and C. Gajate, unpublished observations.

dria in ET-743-treated cells,[4] suggesting that JNK activation could occur downstream of mitochondrial cytochrome *c* release. This is in agreement with recent reports indicating that curcumin, an agent that has been reported to exert both pro- or anti-apoptotic actions (47–54), potentiates mitochondrial cytochrome *c* release (49, 53) and can even induce opening of the mitochondrial permeability transition pore (55). Nevertheless, curcumin, a dietary yellow pigment from *Curcuma longa*, has been reported to affect a great variety of signaling processes in different systems (47–54), and these pleiotropic effects preclude to reach a clear picture of the hierarchical events occurring in curcumin-pretreated cells.

The distinct actions on cell cycle and apoptosis induced by ET-743 suggest that this drug is able to trigger different signal transduction pathways and indicate that ET-743 is able to exert effects on two key processes in cancer treatment, namely cell cycle and apoptosis. Taken together, our data demonstrate the high capacity of ET-743 to stop proliferation and to trigger apoptosis in human cancer cells at very low and pharmacologically relevant doses (ng/ml, nM). Therefore, ET-743 is a promising anticancer drug that can be useful in the treatment of different cancers with either high or low proliferation rates.

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Differential Cytostatic and Apoptotic Effects of Ecteinascidin-743 in Cancer Cells: TRANSCRIPTION-DEPENDENT CELL CYCLE ARREST AND TRANSCRIPTION-INDEPENDENT JNK AND MITOCHONDRIAL MEDIATED APOPTOSIS

Consuelo Gajate, Feiyun An and Faustino Mollinedo

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